Br. J. Pharmacol. (1994), **111**, 1257–1261

Effect of anti-fungal imidazoles on mRNA levels and enzyme activity of inducible nitric oxide synthase

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1 Experiments were performed to examine the effects of anti-fungal imidazole compounds (clotrimazole, econazole and miconazole) on the induction of nitric oxide (NO) synthase and subsequent production of NO in the cultured murine monocyte/macrophage cell line J774 using a specific cDNA probe for inducible NO synthase mRNA and by monitoring nitrite production.

2 Stimulation of J774 cells with lipopolysaccharide (LPS, 10 $\mu g\,ml^{-1}$) resulted in the induction of NO synthase activity as determined by nitrite accumulation in the culture medium (48 ± 3 nmol per 10^6 cells over 24 h). Production of nitrite was inhibited by co-incubation of cells with LPS (10 $\mu g\,ml^{-1}$) and either dexamethasone (10 μM) or N^G-monomethyl-L-arginine (L-NMMA; 0.1 mM), however, only L-NMMA was an effective inhibitor of nitrite production when added after induction of NO synthase had occurred.

3 Co-incubation of J774 cells with LPS (10 $\mu g\,ml^{-1}$) and either clotrimazole, econazole or miconazole (1–10 μM) resulted in a concentration-dependent inhibition of nitrite production over the subsequent 24 h without any evidence for a cytotoxic effect. However, addition of these imidazoles after induction of NO synthase did not inhibit nitrite production.

4 Messenger RNA for inducible NO synthase was not detected in unstimulated J774 cells. Treatment with LPS (10 $\mu g\,ml^{-1}$) for 4 h resulted in significant expression of mRNA for inducible NO synthase which was not altered in the presence of econazole (10 μM) but was reduced significantly by dexamethasone (10 μM).

5 These results demonstrate that anti-fungal imidazoles inhibit the production of nitric oxide by cultured J774 cells by a mechanism which appears to differ from that of dexamethasone and substrate-type inhibitors of NO synthase. Furthermore, the presence of mRNA for NO synthase does not indicate the presence of functionally active NO synthase.

Keywords: Macrophage; lipopolysaccharide; nitric oxide; clotrimazole; econazole; miconazole; imidazoles; anti-fungal agents; dexamethasone; inducible NO synthase

Introduction

Nitric oxide (NO) is a potent and widespread signalling molecule within the cardiovascular, nervous and immune systems (Moncada *et al.*, 1991). In certain tissues, including endothelium and brain, synthesis of NO from L-arginine is catalyzed by the action of a constitutively expressed Ca^{2+}/calmodulin-dependent NO synthase (Palmer & Moncada, 1989; Mayer *et al.*, 1989; Bredt & Snyder, 1990). The NO produced activates guanylate cyclase in target cells. However, in endothelium and many other cells that do not normally synthesize NO (including macrophages), exposure to bacterial endotoxin (lipopolysaccharide, LPS) and/or cytokines, results in expression of a distinct isoform of NO synthase which is often functionally Ca^{2+}/calmodulin-insensitive and makes large amounts of NO (Moncada *et al.*, 1991). In the quantities produced by the inducible enzyme NO is toxic to invading pathogens (Hibbs *et al.*, 1987; Marletta *et al.*, 1988) and certain host cells (Palmer *et al.*, 1992). The two broad groups of NO synthase share certain similarities. One common feature is that they are haemoproteins with homology to cytochrome P450 reductase and possess both oxidase and reductase domains (Bredt *et al.*, 1991; White & Marletta, 1992).

Inhibition of NO synthesis can be achieved in a number of ways. Substrate-based analogues of L-arginine including N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NOARG) compete with L-arginine for metabolism to NO and inhibit the activity of constitutive and inducible enzymes (Moore *et al.*, 1990; Rees *et al.*, 1989; 1990). Alternatively, the expression of the inducible enzyme may be inhibited by glucocorticoids (e.g. dexamethasone and hydrocortisone; Di Rosa *et al.*, 1990; Radomski *et al.*, 1990) which probably act by inhibiting gene expression or the transcription of inducible NO synthase messenger RNA (mRNA). In addition, by inhibiting protein synthesis (Radomski *et al.*, 1990) or production of essential co-factors such as tetrahydrobiopterin (Gross & Levi, 1992), induction of NO synthesis can be inhibited. Recently it has been reported that anti-fungal imidazoles inhibit the activity of constitutive NO synthase through an effect on Ca^{2+}/calmodulin (Wolff *et al.*, 1993a). However, these compounds also inhibit the activity of mammalian cytochrome *P*450 proteins (Rodrigues *et al.*, 1987) by binding to the haem domain. If this interaction also occurred with NO synthase, anti-fungal imidazoles would be expected to inhibit the activity of the Ca^{2+}/calmodulin-insensitive and sensitive isoforms. In the present study we have examined the effects of anti-fungal imidazoles on inducible NO synthase in the murine macrophage cell line J774 and have sought to identify the mechanism of action of inhibition.

Methods

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 10% foetal calf serum, 4 mM L-glutamine, penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$).

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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.

Measurement of nitrite production as an assay of NO release

Production of NO was assayed by measuring the accumulation of nitrite in the culture medium by the Griess reaction (Green *et al.*, 1982; Bogle *et al.*, 1992). J774 cells were seeded into 96-well microtitre plates (10^5 cells per well) and allowed to adhere for 2 h at 37°C following which the medium was removed and replaced with fresh medium containing lipopolysaccharide (LPS, 10 $\mu g\,ml^{-1}$) and either N^G-monomethyl-L-arginine (L-NMMA; 0.1 or 1 mM), dexamethasone (1 or 10 μM) or imidazole compounds (1–10 μM). In some experiments, cells were pre-activated with LPS (10 $\mu g\,ml^{-1}$) for 24 h following which the LPS was removed and cells treated with fresh medium containing either L-NMMA, dexamethasone or anti-fungal imidazoles. Twenty-four hours later an aliquot (100 μl) of the culture medium was removed, mixed with an equal volume of Griess reagent (final concentration: 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 using sodium nitrite as a standard.

Assays of cell viability

Cell viability was measured either by the ability of the cells to exclude the vital stain trypan blue (0.4% w/v in Dulbecco's phosphate buffered saline of composition, mM: NaCl 138, KCl 2.6, Na_2PO_4 8.1, KH_2PO_4 1.5, pH 7.4) as determined by visual microscopy or by assessment of the ability of the cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide; MTT; 0.5 $mg\,ml^{-1}$) into formazan over a 4 h time period according to the method of Mosmann (1983).

Production of cDNA probe for inducible macrophage NO synthase

J774 cells (10^6 cells) were treated with LPS (10 $\mu g\,ml^{-1}$) for 6 h and poly(A)$^+$-selected mRNA was isolated with a Fast-Track mRNA isolation kit according to the manufacturer's instructions (Invitrogen, San Diego, U.S.A.). The mRNA obtained was used for the synthesis of first strand cDNA using random primers with a commercial kit (First Strand, Pharmacia, Milton Keynes, U.K.). The cDNA was then used as a template in a polymerase chain reaction (conditions: 25 cycles of denaturing, 95°C, 1 min; annealing 65°C, 2 min and polymerization 72°C, 1 min), utilizing oligonucleotide primers designed from the published sequence of murine macrophage inducible NO synthase (Primer 1: base pairs 2476–2498 (sense), Primer 2: base pairs 2969–2947 (antisense); Lyons *et al.*, 1992). The derived product was purified by electrophoresis on a low-melting point agarose gel (1% w/v) and subcloned into a plasmid vector (EcoRV linearized dephosphorylated pBluescript II, Stratagene Ltd, Cambridge, U.K.). Subsequently the plasmid was transfected into competent bacteria (XL Blue-1 *E. coli*, Stratagene) and plasmid DNA prepared from bacterial cultures. The identity of the insert was confirmed by double stranded dideoxy sequencing with a kit (Pharmacia). A 493 base pair fragment was isolated by restriction enzyme digestion (BamHI/HindIII, Northumbria Biologicals, Northumberland, U.K.) and used as a probe in Northern blotting.

Total RNA isolation and analysis

J774 cells (10^6 cells in 3 cm^2 wells) were stimulated for 4 h with either medium alone, or medium plus LPS (10 $\mu g\,ml^{-1}$) or LPS in the presence of either econazole (10 μM) or dexamethasone (10 μM). Total RNA was extracted using RNAzol reagent according to the manufacturer's instructions (Biogenesis Ltd, Bournemouth, U.K.). The RNA samples (10 μg quantified from the OD_{260}) were electrophoresed on a formaldehyde-agarose gel and transferred using 20 × SSPE buffer (sodium chloride 3.6 M, sodium phosphate 0.2 M, pH 7.7; disodium EDTA 0.002 M) onto a nylon membrane (Hybond N+, Amersham, Bucks, U.K.) and fixed by exposure to u.v. radiation. The cDNA probe for inducible NO synthase was labelled with [α^{32}P]-dCTP (3000 Ci $mmol^{-1}$, ICN Flow, High Wycombe, U.K.) using a multiprime DNA labelling system (Amersham) according to the manufacturer's instructions. Pre-hybridization was performed as follows, nylon membranes were incubated at 42°C for 1 h with 5 × SSPE, formamide (50% v/v), 5 × Denhardt's solution (bovine serum albumin 0.1% w/v, Ficoll 0.1% w/v and polyvinyl pyrollidone 0.1% (w/v) and sodium dodecyl sulphate (SDS; 0.5% w/v) supplemented with 20 $\mu g\,ml^{-1}$ denatured herring sperm DNA) (Promega, Southampton, U.K.). Following this, membranes were incubated with the ^{32}P-labelled NO synthase probe for 18 h at 42°C in the above buffer minus herring sperm DNA. Buffer was removed and membranes washed twice with 50 ml of 2 × SSPE/0.1% SDS for 15 min at 42°C, then with 50 ml 1 × SSPE/0.1% SDS for 30 min at 65°C. Finally, a high stringency wash was performed with 0.1% SSPE/0.1% SDS for 15 min at 65°C. The membranes were then sealed in plastic wrap and autoradiographed at −70°C using an intensifying screen (Dupont Ltd, Herts, U.K.). As a control for consistency of loading, ribosomal RNA (18S) was quantified by ethidium bromide fluorescence and photography of the formaldehyde-agarose gel before transfer and quantitation by Northern blot analysis.

Materials

All reagents for tissue culture were obtained from either Sigma (Poole, U.K.) or Flow (High Wycombe, Bucks). LPS, phenol-extracted from *Escherichia coli* (serotype 055:B5), clotrimazole, econazole nitrate, miconazole nitrate and dexamethasone were obtained from Sigma. Imidazoles were dissolved to a stock concentration of 2 mM in 100% ethanol (final concentration 0.05–0.5%). N^G-monomethyl-L-arginine acetate was a gift from Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent, U.K.

Data analysis and statistics

Results are shown as the mean ± s.e.mean of n experiments. Statistical analysis was performed by a one-way analysis of variance with $P<0.05$ considered statistically significant. The density of bands on Northern blots and 18S ribosomal RNA bands were quantified with a scanner attached to a computer with image analysis software.

Results

In unstimulated J774 cells, nitrite production over a 24 h period was below the level of detection using the Griess reagent ($n = 6$). Stimulation of cells with lipopolysaccharaide (LPS; 10 $\mu g\,ml^{-1}$) resulted in significant nitrite production (43 ± 3 nmol per 10^6 cells over 24 h, $n = 6$) which was inhibited by co-incubation with either L-NMMA (0.1 or 1 mM) or dexamethasone (1 or 10 μM; Figure 1a). When these agents were added to cells pre-stimulated with LPS

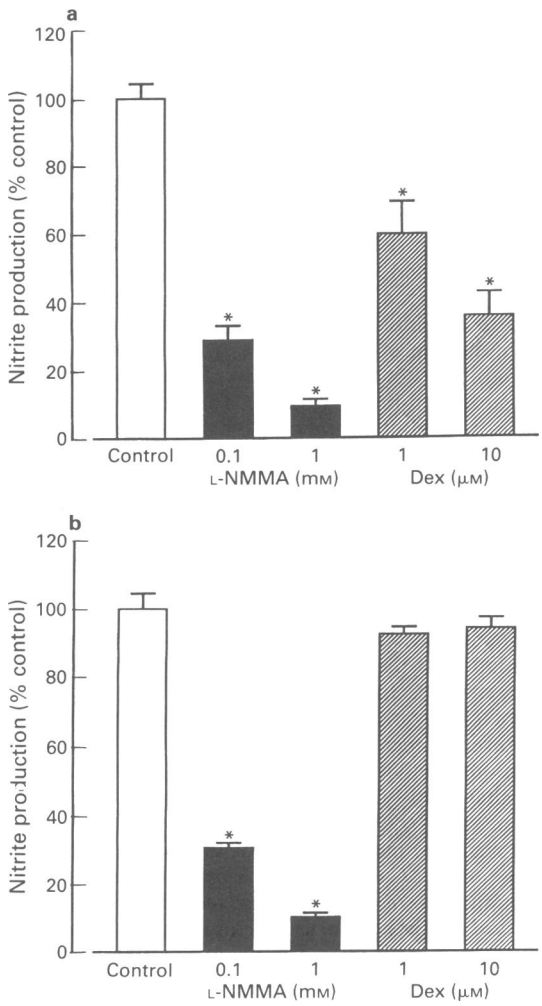

Figure 1 Effects of N^G-monomethyl-L-arginine (L-NMMA) and dexamethasone (Dex) on nitrite production in J774 cells activated with lipopolysaccharide. (a) Cells were stimulated with lipopolysaccharide (LPS, 10 μg ml^{-1}) in the presence of either L-NMMA (0.1 or 1 mM, solid columns) or dexamethasone (1 or 10 μM, hatched columns) and nitrite production was determined 24 h later. (b) Cells were stimulated with LPS (10 μg ml^{-1}) for 24 h after which LPS was removed and cells incubated for a further 24 h in either control medium, or medium containing L-NMMA (0.1 or 1 mM, solid columns) or dexamethasone (1 or 10 μM, hatched columns). Subsequently, nitrite production was measured using the Griess reaction. Values denote the mean ± s.e.mean of replicate measurements in 3 separate experiments; *$P<0.05$ unpaired t test.

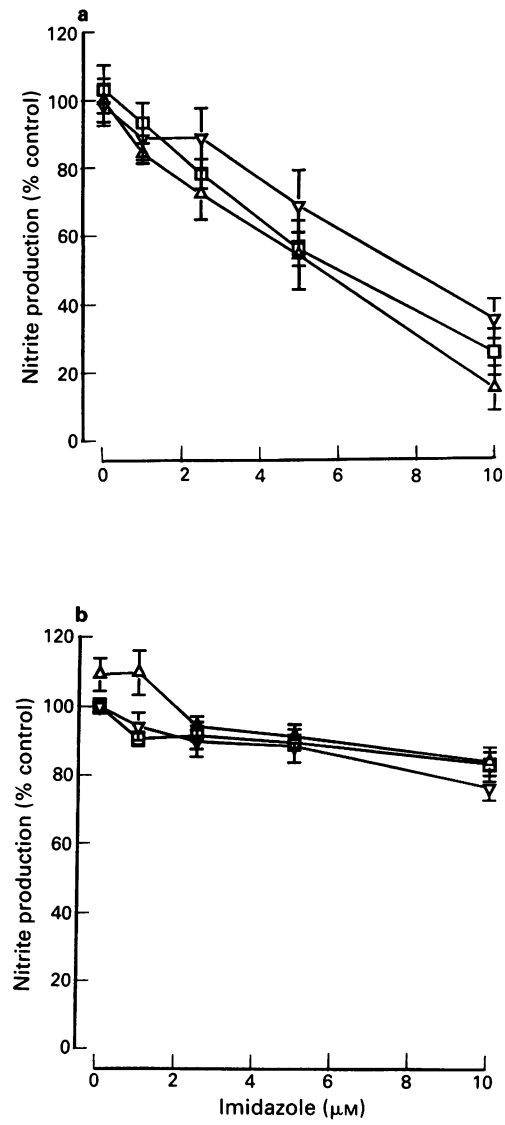

Figure 2 Effects of anti-fungal imidazoles on nitrite production by lipopolysaccharide (LPS)-stimulated J774 cells. (a) J774 cells were stimulated with LPS (10 μg ml^{-1}) for 24 h in the presence of increasing concentrations (1–10 μM) of either clotrimazole (□), econazole (△) or miconazole (▽). (b) J774 cells were pre-stimulated for 24 h with LPS (10 μg ml^{-1}) and then the LPS removed and clotrimazole (□), econazole (△) or miconazole (▽) were added for a further 24 h. Nitrite production was then measured using the Griess reagent. Values denote the mean ± s.e.mean of replicate measurements in 3 separate experiments.

(10 μg ml^{-1} for 24 h), L-NMMA but not dexamethasone caused an inhibition of nitrite production (Figure 1b).

Inhibition of nitrite production by antifungal imidazoles

Clotrimazole, econazole or miconazole (1–10 μM) incubated with J774 cells and LPS (10 μg ml^{-1}) for 24 h produced a concentration-dependent inhibition of nitrite production (Figure 2a, IC_{50} 5.3 ± 0.3, 5.0 ± 0.4 and 7.8 ± 0.7 μM respectively; $n = 3$). In contrast, when cells were pre-activated with LPS (10 μg ml^{-1}, 24 h) and subsequently incubated for 24 h with clotrimazole, econazole or miconazole (1–10 μM) no inhibition of nitrite production was observed (Figure 2b). Ethanol vehicle (0.05–0.5%) did not inhibit nitrite production whether given at the same time as or after LPS ($n = 3$, data not shown). No cytotoxic effects of imidazoles were detected over the concentration-range which inhibited nitrite production (1–10 μM) as assessed by either conversion of MTT to formazan or trypan blue exclusion ($n = 3$ in each case, data not shown).

Effects of antifungal imidazoles on inducible NO synthase mRNA levels

In unstimulated J774 cells, no inducible NO synthase mRNA was observed. However, following stimulation with LPS (10 μg ml^{-1}, 4 h) significant levels of inducible NO synthase mRNA were detected (Figure 3a) and this was associated with an increased nitrite production (Figure 3b). Incubation of cells with LPS (10 μg ml^{-1}, 4 h) in the presence of dexamethasone (10 μM) resulted in a reduced expression of inducible NO synthase mRNA and inhibition of nitrite production (Figure 3a and b). In contrast, treatment of J774 cells with LPS (10 μg ml^{-1}, 4 h) and econazole (10 μM) resulted in levels of mRNA similar to those observed in cells stimulated with LPS alone, whilst production of nitrite was almost completely abolished (Figure 3a and b).

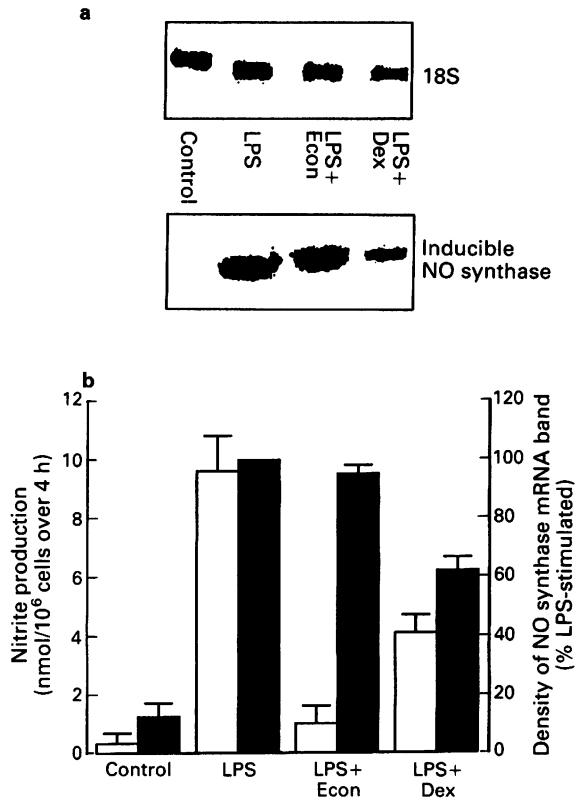

Figure 3 Effects of anti-fungal imidazoles on inducible NO synthase mRNA levels. (a) Representative Northern blot of RNA extracted from J774 cells and hybridized with a cDNA probe specific for murine inducible NO synthase. Cells were incubated for 4 h with either medium alone (control), lipopolysaccharide (LPS, 10 $\mu g\ ml^{-1}$), LPS (10 $\mu g\ ml^{-1}$) and econazole (Econ, 10 μM) or LPS (10 $\mu g\ ml^{-1}$) and dexamethasone (Dex, 10 μM). 18S ribosomal RNA (stained with ethidium bromide) is also shown as a control for consistency of loading. The figure is representative of data obtained in 3 separate experiments. (b) Nitrite production (open columns) and levels of inducible NO synthase mRNA (solid columns) in J774 cells stimulated as described above. Density of mRNA bands, determined by scanning densitometry, is shown as a percentage of that measured in cells stimulated with LPS alone. Results are the mean ± s.e.mean of 3 experiments.

Discussion

The data presented in this study demonstrate that anti-fungal imidazoles inhibit the induction of nitrite synthesis in macrophages. In concentrations similar to those achieved therapeutically (e.g. 2 μM for miconazole; Benson & Nahata, 1988), clotrimazole, econazole and miconazole inhibited nitrite production by J774 cells if added to the cells at the same time as the LPS but were ineffective in cells pre-stimulated with LPS. This profile of action is similar to that of glucocorticoids and might suggest that anti-fungal imidazoles inhibit induction of NO synthase gene expression. However, whereas treatment of J774 cells with dexamethasone resulted in inhibition of mRNA for inducible NO synthase and inhibition of nitrite production, econazole inhibited nitrite production without affecting mRNA levels. These results demonstrate that anti-fungal imidazoles and glucocorticoids inhibit induction of NO synthase activity by different mechanisms. Glucocorticoids appear to inhibit the production of NO before or at the level of transcription while anti-fungal imidazoles inhibit at a stage after mRNA production.

A recent report indicates that anti-fungal imidazoles inhibit the activity of the constitutive NO synthase and this appears to be due to binding and inactivation of the Ca^{2+}/calmodulin complex (IC_{50} <10 μM, Wolff *et al.*, 1993a,b), although at higher concentrations (mM), these drugs may also directly inactivate constitutive NO synthase by interacting with its haem moeity (Wolff *et al.*, 1993b). In the present study, anti-fungal imidazoles inhibited the activity of the inducible NO synthase if given at the same time as the stimulus for enzyme induction (LPS) but were ineffective when given after the enzyme was induced. These results are unlikely to be due to a cytotoxic effect of the compounds since this would be expected to be similar whether the drugs were added before or after induction of NO synthase. More importantly, assays of cell viability showed no adverse effects of imidazoles over the concentration-range studied. The failure of anti-fungal imidazoles to inhibit enzymic activity of the expressed inducible NO synthase is not unexpected since this enzyme type is functionally Ca^{2+}/calmodulin-independent although it is known to bind the Ca^{2+}/calmodulin complex tightly (Cho *et al.*, 1992). Similarly, although imidazoles are reported to inactivate the haem-moiety of enzymes this only occurs at concentrations 50–100 fold higher than those used in the present study (Wolff *et al.*, 1993a,b).

One possible mechanism of action is that imidazoles inhibit the translation of the inducible NO synthase mRNA into NO synthase enzyme. Interaction of imidazoles with the haem moiety of NO synthase at a vital stage of protein assembly might prevent dimerization of the enzyme which is necessary for functional activity (Baek *et al.*, 1993). Alternatively, it is known that anti-fungal imidazoles bind Ca^{2+}/calmodulin (Hegeman *et al.*, 1993) and thereby through this mechanism could prevent the incorporation of this complex into the NO synthase enzyme. Calcium itself appears to be less important in the induction of NO synthase activity; although Ca^{2+}-channel antagonists such as nifedipine inhibit induction of NO synthase in J774 cells (Szabo *et al.*, 1993), other Ca^{2+}-channel antagonists such as verapamil and diltiazem do not (Hauschildt *et al.*, 1990; Szabo *et al.*, 1993). Further experiments will be required to determine the precise mechanism by which anti-fungal imidazoles inhibit the induction of NO synthase activity in J774 cells.

The results of this study demonstrate that anti-fungal imidazoles inhibit the induction of functionally active NO synthase in J774 cells through a mechanism that differs from other known inhibitors of induction including glucocorticoids and actinomycin D which are likely to act via inhibition of gene expression and transcription respectively. The results obtained with anti-fungal imidazoles give some insight into the process of induction of NO synthase activity and coupled with previous reports suggest that Ca^{2+}/calmodulin may play an important role in regulating the activity of the inducible isoform of NO synthase. Furthermore our results indicate that it is possible to produce levels of mRNA for inducible NO synthase without production of NO. This finding has implications for studies in which the level of NO synthase mRNA are taken as an indication of functional activity. It remains to be determined how our study, and other studies demonstrating that imidazoles inhibit the activity of the constitutive NO synthase (Wolff *et al.*, 1993a) contribute to the reported anti-inflammatory (Petri *et al.*, 1986) and hypertensive effects of these and other imidazole drugs.

Note added in proof

Whilst this paper was in press, Yu & Tomasa (*Crit. Care Med.* (1993), **21**, 1635–1642) reported that ketoconazole reduced mortality in patients with septic shock.

This work was supported by the Wellcome Trust. We are grateful to Dr Salvador Moncada for the gift of L-NMMA.

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(Received November 4, 1993
Revised December 6, 1993
Accepted December 9, 1993)